Algal Research 79 (2024) 103480

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Unwrapping the (glyco-)lipidome in the microalgae *Microchloropsis gaditana:* Effects of eco-friendly extraction methods

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ARTICLE INFO

Keywords:
Microchloropsis gaditana
Green extraction approaches
LC-MS/MS
Lipidomics
Glycolipids
Phospholipids

ABSTRACT

Microalgae have recently captivated worldwide attention because of their extraordinary lipid composition, which includes essential omega-3 and omega-6 long-chain polyunsaturated fatty acids (LC-PUFA), neutral lipids, and more complex lipids (glycolipids and phospholipids), as well as eicosanoids. To explore this further, we present a novel (glyco-)lipidomics strategy, including a green chemistry extraction approach, liquid chromatography high-resolution mass spectrometry (LC-HRMS) lipid profiling, and a comprehensive data evaluation workflow. Using untargeted lipidomics based on LC-HRMS approaches, we studied the lipid composition up to the molecular species level of the microalgae *Microchloropsis gaditana* (formerly *Nannochloropsis gaditana*), focusing on the distribution of saturated, monounsaturated, and polyunsaturated fatty acids in different lipid classes. Our analysis successfully identified 520 lipid molecular species from 18 lipid classes. Regarding fatty acid composition, our primary focus was on elucidating the distribution of LC-PUFA across the different microalgal lipid classes. This particular strategy holds potential as it may unravel the use of microalgal lipids as innovative sources of food ingredients. Our findings show that LC-PUFA are relatively elevated in membrane lipids, in which cholesterol esters (ChE) have the highest PUFA share (94 %), followed by polar membrane lipids (glycolipids and phospholipids), with phosphatidylethanolamines (PE) up to 67 %, phosphatidylglycerols (PG) up to 47 %, and monogalactosyldiacylglycerols (MGDG) up to 47 % PUFA content. This highlights the high percentage of PUFA in the structural parts of microalgae and the importance of these lipid classes as a reservoir for PUFA. Finally, we also compared a classical Folch extraction method with environmentally friendly ultrasound-assisted extraction approaches and enzymatic pre-treatments to investigate the lipid composition and enrichment of valuable lipid classes. Thus, this extensive analysis of the (glyco-)lipidome of *Microchloropsis gaditana* provides valuable insights into the existing knowledge about this microalgae species, enhancing our understanding of the complex lipid composition and opening up new opportunities for potential applications in the biotechnology and food industry.

1. Introduction

Microalgae are crucial sources for meeting the global population's needs in terms of more sustainable food supplies, specifically concerning lipid demand. Microalgae produce a wide range of high-value lipids, including omega-3 and omega-6 long-chain polyunsaturated fatty acids (ω-3 and ω-6 LC-PUFA), neutral lipids, and more complex lipids, which have essential roles in cell structure and biological functions [1]. *Microchloropsis gaditana* (L.M. Lubián, formerly *Nannochloropsis gaditana*) is a species of the genus *Microchloropsis* (Monodopsidaceae, Eustigmatophyceae) mainly found in marine environments accumulating high lipid contents (up to 30 % of the total biomass) [2]. Due to the exceptional lipid content, especially polyunsaturated fatty acids (PUFA), and their high growth rates, *Microchloropsis* microalgae are currently being investigated for the use as nutritional supplements and functional foods [3]. Among the diverse microalgae species within this genus, *M. gaditana* is one of the most promising microalgae species for producing valuable lipids with excellent lipid composition containing elevated levels of ω-3 LC-PUFA, mainly eicosapentaenoic acid (EPA, 20:5) [4]. As a primary producer of essential PUFA, *M. gaditana* is cultivated in laboratory and pilot plant facilities, with a continuous focus on scaling up the process for the food industry [5]. It has been

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https://doi.org/10.1016/j.algal.2024.103480
Received 27 July 2023; Received in revised form 22 January 2024; Accepted 14 March 2024
Available online 15 March 2024

proven that PUFA biosynthesis in microalgae can be altered by different cultivation conditions. For example, nitrogen deprivation and organic carbon availability in the media of the microalgae can enhance the production of fatty acids [6,7].

Knowing about the distribution of PUFA in different lipid classes and cell compartments could be the key to applying microalgal lipids as novel sources of food ingredients. Omega-3 and omega-6 LC-PUFA are essential fatty acids, meaning that they cannot be produced by human biosynthesis and, therefore, have to be provided through dietary sources [8]. For example, n-3 PUFA, such as EPA and DHA, have important functions for processes in the brain, such as anti-inflammatory activities as well as neurotransmission and synaptic plasticity [9]. However, they can only cross the blood-brain barrier in the phospholipid form and not as triglycerides [10–12]. In addition to phospholipids, which are the main building blocks of membranes in humans and yeast, glycoglycerolipids are important membrane components in plants, eukaryotic algae, and cyanobacteria [13]. Glycoglycerolipids, such as monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), or sulfoquionovosyldiglycerols (SQDG), show a diverse profile of critical biological functions. For instance, they are involved in signaling and regulation processes and exhibit anti-tumor and anti-inflammatory effects [14–16].

Lipidomics aims for a comprehensive characterization and the generation of an as complete as possible picture of the lipidome of a cell, tissue, or organism [17]. Some of which were extensively reviewed in marine organisms, from sampling to data processing, by Rey et al. [18]. These lipidomic fingerprints may serve to understand biochemical processes and membrane characteristics, chemotaxonomy, or even elucidate the health benefits of microalgal lipids in humans. In particular, beneficial dietary aspects of microalgae can be better understood by studying lipid metabolism and functions [19].

Due to their thick and firm cell wall built as a bilayer with an inner cellulose and an outer hydrophobic layer, the cell disruption and extraction of the lipids from *M. gaditana* is an ongoing challenge [20]. Widely used extraction methods for lipids from marine organisms are the classical chloroform-based Bligh and Dyer [21] or the Folch [22] extraction but also as newer methyl-*tert*-butylether (MTBE):methanol approaches [23]. Recently, the need for more environmentally friendly extraction methods has been emphasized, especially in food chemistry and toxicology. Eco-friendly strategies are based on advanced extraction techniques, including pressurized liquids, ultrasound-assisted extraction (UAE), or enzyme-assisted extraction (EAE), combined with green solvents [1,23,24]. Castejón et al. recently introduced an innovative and eco-friendly approach for extracting *M. gaditana* lipids that combines enzymatic pre-treatment with UAE and ethanol as a solvent [4]. This new approach represents a novel and environmentally friendly alternative to the conventional Folch method, substituting the use of toxic chloroform-methanol mixtures with ethanol.

The objective of the current study was to provide a comprehensive overview on the lipidome of *M. gaditana* through the implementation of environmentally sustainable extraction techniques. Gas chromatography mass spectrometry (GC–MS) is the gold standard for fatty acid profile determination leading to summed parameters, which enables quick quantitative comparisons of different sample sets [25,26]. Previous studies using GC–MS analysis revealed high contents of PUFA [1,4] in *M. gaditana,* preeminently 20:4 *n*-6 and 20:5 *n*-3, highlighting the value of this microalga as an alternative essential fatty acid source. However, GC–MS is not capable of providing detailed structural information on lipid class (head group), lipid species (fatty acid composition with carbon number and double bonds) or molecular lipid species level [27] (resolving all structural subunits e.g. lipid class and fatty acid composition). However, liquid chromatography mass spectrometry (LC-MS) can resolve structural details such as different lipid classes or species. Therefore, LC-MS enables intact lipid analysis without the need of hydrolysis before methylation derivatization in GC–MS analysis.

Using novel (glyco-)lipidomics workflows, we aimed to unlock the lipidome of *Microchloropsis gaditana* and investigated the distribution of highly valuable PUFA among the different lipid classes. Furthermore, we assessed the impact of eco-friendly extraction methods on the (glyco-) lipidomic profile, intending to evaluate their potential as greener alternatives for producing lipid-rich microalgal extracts of nutritional importance.

2. Material and methods

2.1. Materials & chemicals

The lyophilized microalgal biomass of *Microchloropsis gaditana* (batch L3250520) was purchased from Cianoalgae SI (Gipuzkoa, Spain). Enzymatic solutions (Viscozyme® L, Celluclast® 1.5 L, Saczyme® Yield) were kindly provided by Novozymes A/S (Bagsvaerd, Denmark). The internal standards mixture (EquiSPLASH™ LIPIDOMIX® Quantitative Mass Spec Internal Standard) and C16 Lactosyl(ß) Ceramide (d18:1/16:0) (D-lactosyl-ß-1,1′N-palmitoyl-D-erythro-sphingosine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA). Chloroform, methanol, and ethanol for the extraction of the crude lipids were purchased from Fisher Scientific GmbH (Vienna, Austria). All solvents for analysis were of LC-MS grade. Isopropanol (IPA), water, methanol, and acetonitrile were obtained from Honeywell (Seelze, Germany). Formic acid was purchased from VWR (Radnor, Pennsylvania, USA) and ammonium format from Sigma Aldrich (St. Louis, Missouri, USA).

2.2. Extraction of crude lipids

Crude lipids from *M. gaditana* microalgal biomass were extracted using the traditional Folch method [22] and an innovative and eco-friendly approach recently developed by Castejón et al. [4].

2.2.1. Folch extraction

1 g of dry microalgal biomass was extracted with 20 ml chloroform: methanol (2:1) and vortexed for 2 min. The samples were centrifuged at 300 rpm for 10 min, and the organic layer was collected. The extraction step was repeated 3 times over the same microalgal biomass. The organic layers were combined and washed with water. After centrifugation at 4000 rpm for 10 min, the organic chloroform layer containing the lipids was separated. The solvent was evaporated in a rotary evaporator (Heidolph Hei-Vap Value HB/G3, Schwabach, Germany) under reduced pressure at 40 °C. The crude lipids were stored at −20 °C until further processing.

2.2.2. Ultrasound-assisted extraction

Briefly, 1 g of dry microalgae was dispersed into ethanol at a ratio of 1:10 (*w/v*). The ultrasound treatment was performed in an ultrasound bath (Elmasonic P 30H, Elma Schmidbauer GmbH, Singen, Germany) using an ultrasound frequency of 37 kHz and an ultrasonic power of 100 W at a temperature of 50 °C for 30 min. After that, samples were filtered using a 0.2 µm Claristep® filter (Sartorius, Göttingen, Germany), and the solvent was evaporated and the extract stored at −20 °C.

2.2.3. Enzymatic pre-treatment

Different pre-treatments of the microalgal biomass using commercially available enzymes (Viscozyme® L, Celluclast® 1.5 L, Saczyme® Yield) and a mix of the enzymatic solutions (1:1:1 (*v/v*)) were tested. 1 g of dry microalgal biomass was dispersed into 10 ml acetate buffer (pH 4.5), and the respective enzyme was added. An incubation step of 1 h using an Eppendorf Thermomixer C (Hamburg, Germany) at 50 °C under constant shaking at 500 rpm followed. The pre-treated samples were centrifuged, and the enzymatic solution was removed. Then, the pre-treated microalgal biomass was further treated using the UAE technique, as described in Section 2.2.2.

2.3. Sample preparation for analysis

To 10 mg of crude microalgal lipid extract, 900 µl methanol and 10 µl of the 1:10 pre-diluted internal standard mixture (EquiSPLASH™, C16 Lactosyl(β) Ceramide) were added, and the solution was vortexed. After adding 3 ml MTBE, the mixture was incubated under constant shaking for 1 h. Phase separation was induced by adding 750 µl water. The extracts were incubated for 10 min and centrifuged at 1000 rpm. The organic layer was collected, and a second extraction using 1.395 ml MTBE/methanol/water (10:3:2.5, *v/v/v*) was performed. Both organic phases were combined, the solvent evaporated, and the samples re-suspended in 1 ml IPA/H_2O (65:35; v/v).

2.4. Instrumental

Global (glyco-)lipidomics profiling of bulk lipids in *M. gaditana* was accomplished with RP-LC and an electrospray ionization data-dependent MS/MS (ESI-DDA-MS/MS) method. Chromatography was performed on a Vanquish Horizon HPLC (Thermo Fisher Scientific, Waltham, MA, USA) system using an Acquity HSS T3 (2.1 mm × 150 mm, 1.8 µm) (Waters, Milford, MA, USA) column protected with a VanGuard Pre-column (2.1 × 5 mm, 100 Å, 1.8 µm). Mobile phase A was ACN/H_2O (3:2; *v*/v), and mobile phase B IPA/ACN (9:1; v/v), both containing 0.1 % formic acid and 10 mM ammonium formate. The column temperature was 40 °C, and the flow rate was 250 µl/min throughout the gradient. The following gradient was applied: 0–8 min ramp from 55 % B to 65 %, 8–13 min ramp from 65 % B to 85 % B, 13–15 min ramp from 85 % B to 100 % B followed by isocratic elution with 100 % B (15–20 min), fast switch to 55 % B and equilibration with starting conditions for 3 min. The HPLC system was coupled to a Thermo Scientific™ Orbitrap ID-X™ Tribrid™ Mass Spectrometer using ESI in both positive and negative ion mode. ESI source parameters were set as follows: spray voltage 3500 V in positive and 2900 in negative ion mode, sheath gas 40, auxiliary gas 8, ion transfer tube temperature 275 °C (positive mode) and 300 °C (negative mode). For lipid identification, a sample pool including all microalgae extractions and an extraction blank were measured in iterative steps to perform in-depth lipid profiling by automated exclusion lists (based on successful MS2 fragmentation) using the Thermo Scientific AcquireX data acquisition. Fragmentation was achieved using an HCD collision energy of 25 % in positive and 28 % in negative ion mode and a normalized AGC target of 150 % (positive) and 100 % (negative), respectively. For the relative quantitation, spectra of the replicates from the different extractions were acquired using the same method but in ESI positive full MS mode at a resolution of 120,000.

2.5. Data processing

Data-dependent MS/MS spectra were matched against in silico fragments using LipidSearch 5.0 software with an absolute intensity threshold of 10,000 on MS1 level and a maximum mass error of 5 ppm on MS1 and 8 ppm on MS2 level. The detailed LipidSearch parameters and the filter criteria are listed in Table SI1 and Figure SI1 of the supplementary material. Relative quantification on MS1 level was performed in Skyline by extraction of the mass of the most prominent ion ($[M + H]^+$, $[M - H]^-$ or $[M + NH_4]^+$), allowing 5 ppm mass tolerance. Peak areas were imported into R for subsequent statistical analysis. Peak areas on MS1 level were normalized to the dry weight of the samples and the peak areas of the deuterated corresponding lipid class or a chemically similar lipid class contained in the class-specific internal standard (EquiSPLASH™). Results are given as peak area per mg dry weight and area of the class-specific internal standard. Each extraction was performed in triplicates, and the results were subjected to statistical analysis using R, including one-way ANOVA and following Fisher LSD post-hoc test (significance level of $p < 0.05$). The results are presented as the mean of the 3 replicates ± standard deviation.

The schematic representation of the full lipidomics workflow used in the study is shown in Fig. 1.

3. Results and discussion

3.1. Overview of the (glyco-)lipidome of M. gaditana

Using an untargeted lipid profiling LC-MS approach, 520 lipid molecular species from 18 lipid classes namely ceramides (Cer), cholesterol esters (ChE), diglycerides (DG), digalactosyldiacylglycerols (DGDG), hexosylceramides (Hex1Cer), bisoleoyl-lysobisphosphatidic acids (LBPA), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), lysophosphatidylinositols (LPI), monogalactosyldiacylglycerols (MGDG), phosphatidylcholines (PC), phosphatidylethano lamines (PE), phosphatidylglycerols (PG), phosphatidylinositols (PI), phosphatidylserines (PS), sulfoquinovosylmonoglycerols (SQMG), sulfoqionovosyldiglycerols (SQDG) and triglycerides (TG) were identified in samples of *M. gaditana* (the structures of the lipid classes are presented in Figure SI2 of the supplementary material). Fig. 2 depicts the number of identified species per lipid class. Each lipid species was identified in a pooled sample using ddMS/MS and further detected on MS1 level in every sample regardless of the extraction method. TG represents the most dominant lipid class in terms of the number of different species. A variety of PC, DG, Cer, DGDG, and MGDG species and minor amounts of other phospholipids, lysophospholipids, and ChE were identified in each replicate and every *M. gaditana* extract (Folch extraction, UAE extraction, enzyme pre-treated extracts). Adducts, *m/z* values, and retention times for all identified lipids are listed in Table SI2 of the supplementary material.

Polar lipids, specifically glycolipids and phospholipids, play a crucial role in signaling processes and constitute a substantial part of the structural components of microalgae cells. They are responsible for dividing the cell into distinct compartments and building the cell membrane [28,29]. Although we cannot determine the actual location of the lipids using the applied extraction techniques and LC-MS method, we can safely assume that most glycolipids and phospholipids originate from different membrane fractions within *M. gaditana* samples. Overall, we found that these glycolipids comprise a large fraction of the total lipids in *M. gaditana* (DGDG 12 %, MGDG 17 % of the total lipid amount). 28 different DGDG, 26 MGDG, 1 SQMG, and 11 SQDG were identified on lipid species level in this microalgae species. MGDG (14:0_20:5), DGDG (16:0_16:1), and DGDG (16:1_20:5), detected as $[M + NH_4]^+$ ions, were the most abundant glycolipid species detected in the samples. Specific fragments for the glycolipid classes DGDG and MGDG are neutral losses of the respective fatty acid and H_2O [NL: FA + 18]. SQDG and SQMG were detected as $[M - H]^-$ ions with the most abundant species being SQDG (16:0_16:1). The other major group of polar membrane lipid classes was phospholipids (27 % of the total lipid amount), where the most abundant molecular species are PC (16:0_18:2) and PC (16:1_18:2). Especially PEs and PGs, which are an important part of the thylakoid membrane in microalgae [30], contain high amounts of long-chain PUFA in the form of PE (20:4_20:4), PG (16:0_20:5) or PG (22:6_20:4) for example.

The lipidome of *M. gaditana* has not been described in such detail yet, especially in terms of fatty acid compositions within different lipid classes (see Section 3.2.). In a previous study by Cauchie et al., the first lipidome profile of this microalgae was reported on the structural (polar) lipidome [31]; however, in this study, we expand it to include the neutral storage lipids, providing a comprehensive lipidomic profile of *M. gaditana*. Other LC-MS studies of the polar on the polar lipidome of *Nannochloropsis oceanica* and *limnetica* identified the same types of phospho- and glycolipids in *Nannochloropsis* sp. [32,33].

3.2. Fatty acid distribution among M. gaditana lipid classes

Understanding the specific lipid classes to which PUFA are linked is crucial for elucidating their biological functions. The health benefits of

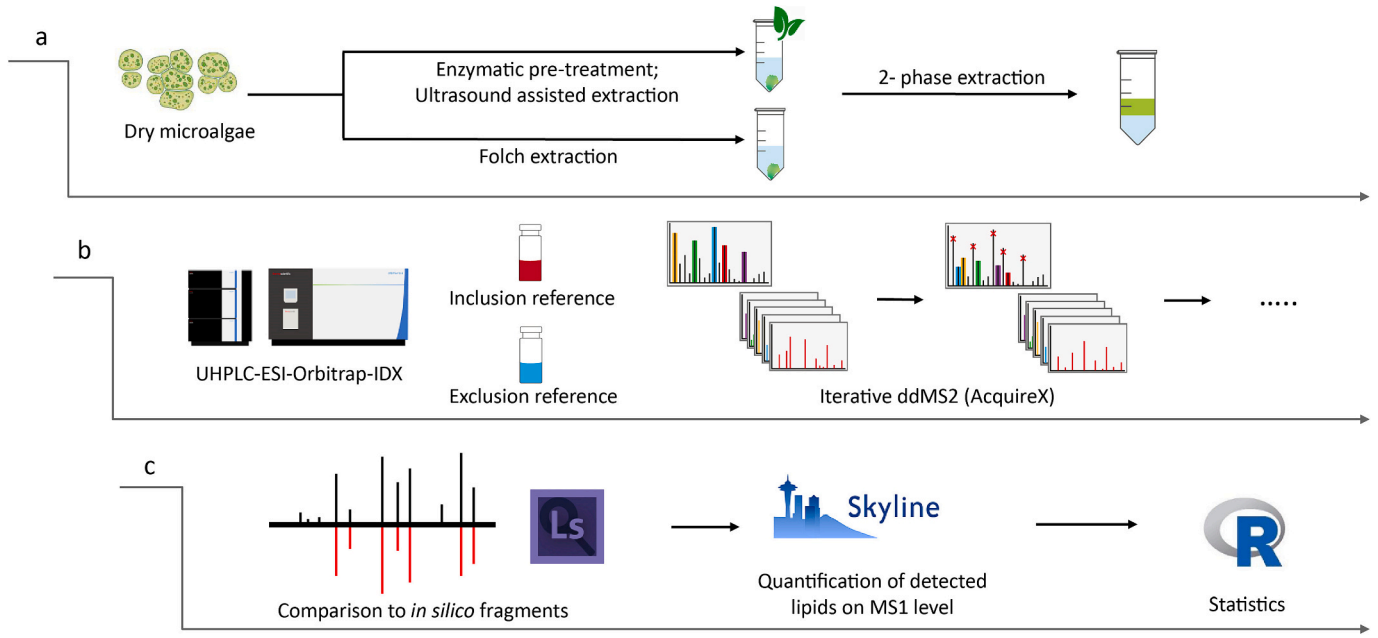

Fig. 1. Schematic depiction of the lipidomics workflow: a) traditional Folch method and eco-friendly extraction approaches were applied to the dry microalgal biomass, b) the lipid extracts were measured using an iterative ddMS2 acquisition method on an UHPLC-ESI-Orbitrap-IDX instrument and c) lipids were identified using LipidSearch, relatively quantified using Skyline and subjected to statistical analysis.

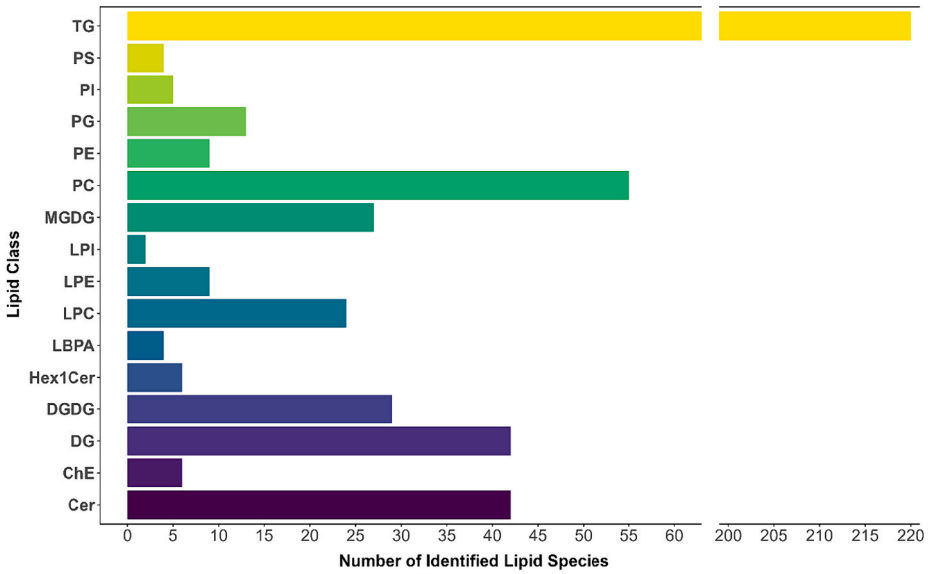

Fig. 2. Number of identified lipid species per lipid class. Each lipid species was identified using iterative data-dependent MS/MS (ddMS/MS) in a pooled sample and then detected on MS1 level in every sample regardless of the extraction method (traditional Folch extraction, UAE extraction with ethanol, UAE extraction with ethanol including pre-treatment with either Viscozyme, Celluclast, Saczyme or an enzyme mix) and in each of 3 biological replicates. Identification was on lipid species level (= level D; known functional groups) according to the metabolomics society [28,29]. Cer = ceramides, ChE = cholesterol esters, DG = diglycerides, DGDG = digalactosyldiacylglycerols, Hex1Cer = hexosylceramides, LBPA = bisoleoyl-lysobisphosphatidic acids, LPC = lysophosphatidylcholines, LPE = lysophosphatidylethanolamines, LPI = lysophosphatidylinositols, MGDG = monogalactosyldiacylglycerols, PC = phosphatidylcholines, PE = phosphatidylethanolamines, PG = phosphatidylglycerols, PI = phosphatidylinositols, PS = phosphatidylserines, TG = triglycerides.

PUFA are highly dependent on the biological context in which they exhibit their effects [34,35]. Fig. 3 shows the relative contents of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFA in the most abundant lipid classes given as an average over all samples and replicates.

As one of the significant membrane lipids [36], cholesterol esters (ChE) have the highest PUFA share of 94 % in the *M. gaditana* samples. The investigated PUFA are further elevated in polar membrane lipids like glycolipids and phospholipids up to 67 % in PE, 47 % in PG, and 47 % in MGDGs underlining the high occurrences of PUFA in the structural parts of microalgae and the importance of these lipid classes as a reservoir for PUFA [37].

Our results on PUFA contents in *M. gaditana* correspond to previous studies, showing that microalgal polar lipids (glycolipids and phospholipids) are rich in PUFA [38,39]. EPA (20:5), for example, is transferred back to the chloroplast after biosynthesis, where it attaches to glycolipids or is further synthesized into phospholipids [40]. The non-polar lipids contain minor amounts of PUFA but accumulate SFA and MUFA

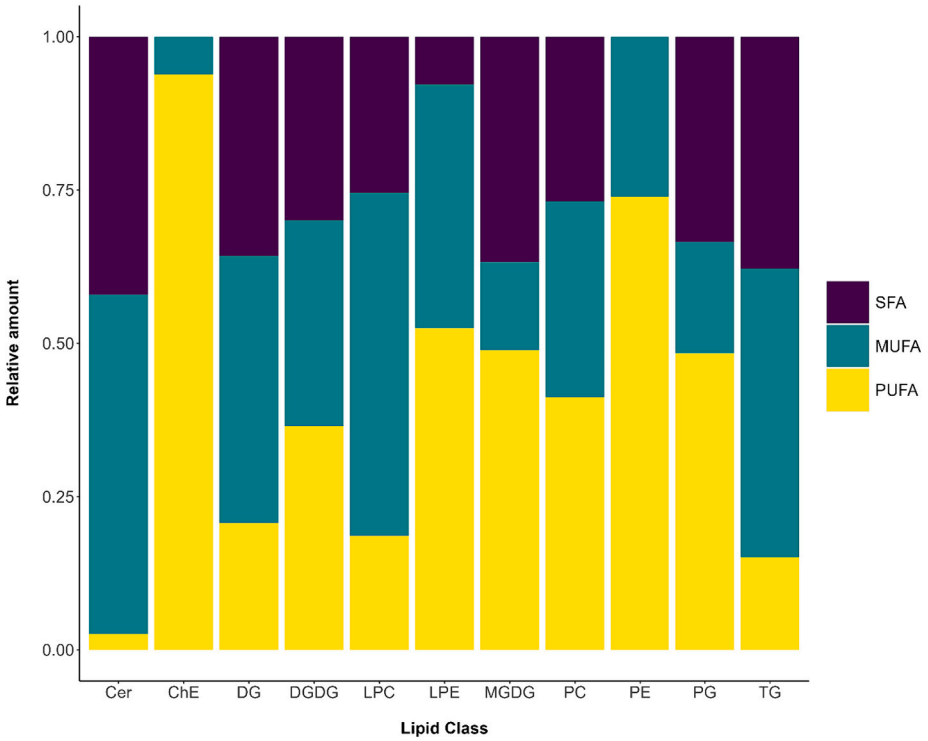

Fig. 3. The mean share of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) in the different lipid classes of *M. gaditana* (all different replicates and samples). The share [%] refers to the sum of area ratios (normalization to the dry weight of the crude lipids and the internal standard (ISTD)) of the SFA/MUFA/PUFA-containing lipid species in relation to the sum of area ratios (normalization to the dry weight of the crude lipids and ISTD) of all lipids within one lipid class. The number of fatty acids attached to one backbone depends on the lipid class and is considered via factors (1, 2, or 3). Cer = ceramides, ChE = cholesterol esters, DG = diglycerides, DGDG = digalactosyldiacylglycerols, LPC = lysophosphatidylcholines, LPE = lysophosphatidylethanolamines, MGDG = monogalactosyldiacylglycerols, PC = phosphatidylcholines, PE = phosphatidylethanolamines, PG = phosphatidylglycerols, PI = phosphatidylinositols, TG = triglycerides.

[41]. Fig. 3 reveals that Cer exhibit the highest relative levels of SFA and MUFA among the studied lipid classes. Moreover, DG, TG, and LPC accumulated substantially higher SFA and MUFA ratios than the other lipid classes.

The detailed fatty acid profiles depend on the lipid class and vary between polar and non-polar lipid classes. Fig. 4 shows the fatty acid distribution with the highest abundant fatty acids across the different lipid classes. Fatty acid methyl ester (FAME) profiles via GC–MS of *N. oceanica, N. limnetica,* and *M. gaditana* have been described by other authors and show similar fatty acids in different ratios among the *Nannochloropsis* species [1,3,42–44]. Our results from *M. gaditana* show that the glycolipids DGDG and MGDG contain relatively high amounts of 20:4 and 20:5 fatty acids, while PC are rich in 18:1 and 18:2 fatty acids and TG in 16:0 and 16:1 fatty acids. Of the essential 20 carbon chain fatty acids, 20:4 mostly occurs in PE, where it accounts for 57 % of the total fatty acid content, whereas 20:5 has the highest abundance in ChE (44 %), DGDG (31 %), MGDG (34 %), LPE (45 %) and PG (38 %). In accordance with previously reported GC–MS methods [1], most detected fatty acids in *M. gaditana* were long-chain fatty acids, mostly with an even number of carbon atoms such as 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 20:4 and 20:5. Recent GC–MS studies by Castejón et al. also revealed the presence of omega-6 (20:4, arachidonic acid) and omega-3 (20:5, eicosapentaenoic acid) fatty acids in *M. gaditana* [4]. Odd-chain fatty acids are only present in small amounts and mainly as one of the three fatty acids bound to the glycerol backbone of TG or as the long-chain base in Cer (data not shown in Fig. 4). Most PUFA occur as 20 carbon chains containing fatty acids such as 20:4 and 20:5. Notably, no saturated fatty acids were detected in ChE and PE, indicating a distinct fatty acid profile for these lipid classes. For instance, ChE(20:5) was found to be the most abundant form of PUFA in ChE, followed by less abundant forms such as ChE(18:2), ChE(22:5), ChE(20:4), and ChE (20:3). Unlike other microalgae genus which mainly have ergosterol, stigmasterol, or chondrillasterol as major phytosterols, *Nannochloropsis* microalgae mostly contain cholesterol and the respective esters [25]. Storage lipids such as DG, TG, and Cer contained relatively high amounts of SFA and MUFA such as 16:0, 18:0, 16:1, and 18:1. The most abundant lipid species in the group of storage lipids were DG (16:0_16:1), TG (16:0_16:1_16:1) and TG (16:0_16:0_16:1).

These results are in accordance with previous results from *Nannochloropsis oculata,* which demonstrate high PUFA contents in the polar lipids, especially phospholipids and glycolipids, in the microalgae [45]. Fatty acid analysis of TG fractions, on the other hand, revealed that TG contain relatively high amounts of SFA in the TG fractions of *Nannochloropsis* sp. [46,47]. Using the presented RP-MS/MS method, we are able, for the first time, to provide a comprehensive overview of the *M. gaditana* (glyco-)lipidome, including not only fatty acid content information but also detailed lipid and glycolipid class compositions up to the molecular lipid species. Combining retention time, accurate mass, and fragment information enabled us to report the highest number of lipids in microalgae.

3.3. Impact of the eco-friendly extraction methods on the M. gaditana lipid profile

The extraction of lipids from microalgae poses a significant challenge due to the dense and rigid nature of their cell walls [23]. Successful extraction approaches involve the disruption of the cell wall without compromising environmental and health safety. In this sense, we compared different green extraction strategies using ultrasound and enzymes to evaluate their effectiveness in extracting lipids from the microalgae *M. gaditana*. Castejón et al. successfully optimized extraction conditions (incubation times and temperatures) to achieve the highest

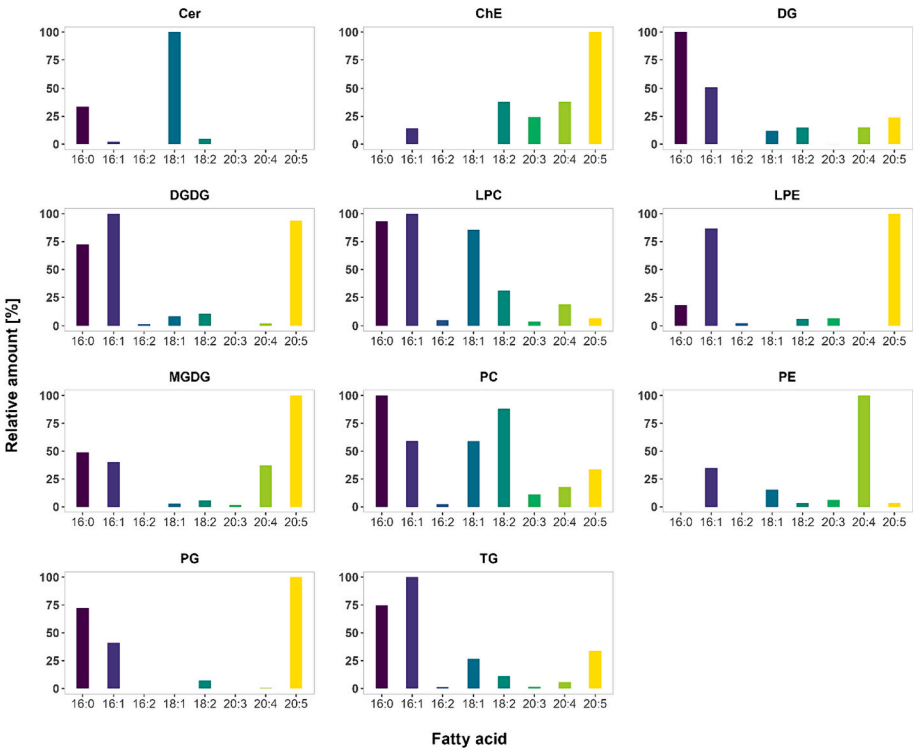

Fig. 4. The average abundance of the 8 most abundant fatty acids is plotted for each lipid class over all replicates and samples. The sum of the peak areas is normalized by the dry weight and the peak area of the class-specific internal standards and then plotted as a relative value to the most abundant fatty acid within the lipid class. Abbreviations: Cer = ceramides, ChE = cholesterol esters, DG = diglycerides, DGDG = digalactosyldiacylglycerols, LPC = lysophosphatidylcholines, LPE = lysophosphatidylethanolamines, MGDG = monogalactosyldiacylglycerols, PC = phosphatidylcholines, PE = phosphatidylethanolamines, PG = phosphatidylglycerols, PI = phosphatidylinositols, TG = triglycerides.

possible lipid yield while meeting environmental and health requirements [4]. To further explore the impact of these eco-friendly approaches on the lipidome profile of *M. gaditana*, Fig. 5 displays the lipid amounts detected in the main lipid classes for each greener approach compared with the conventional Folch method.

The total extracted lipidome (Fig. 5, bottom right side) was higher for all the environmentally friendly extraction methods tested compared to the conventional Folch extraction (UAE 185 %, Saczyme 155 %, Viscozyme 138 %, Celluclast 154 %, enzyme mix 157 %; $p < 0.05$), with the exception of Viscozyme, which yielded a comparable total lipidome to the traditional method ($p > 0.05$). The highest total lipid yield was obtained by the UAE approach (without enzymatic pre-treatment) with a factor 2 increase compared to the Folch extraction. This finding underscores the effectiveness of the greener methods compared to the Folch extraction. However, these results contrast the lipid yield (determined gravimetrically) reported by Castejón et al. [4], where the Folch method and Saczyme exhibited the highest extraction efficiency. Even the gravimetrically determination of lipids is widely used for evaluating the efficiency of an extraction process; the extracts can be contaminated due to the co-extraction of other lipophilic compounds (i.e., carotenoids), creating then a discrepancy between gravimetric and analytical methods (as is the case in the present study).

Upon closer investigation of the lipid classes, we can observe a fractionation or enrichment of lipids based on the extraction method, with particular emphasis on the type of enzyme used. Focusing on neutral lipids, namely TG and DG, a positive effect of the alternative methods is evident. The use of only ultrasound for the extraction of TG shows a significant improvement ($p < 0.05$ compared to the Folch method). In contrast, the use of Celluclast proves to be a promising alternative for increasing the quantity of DG in microalgal extracts. In terms of polar lipids, the use of ultrasonic and enzymatic approaches significantly impacted the recovery of phospholipids and lysophospholipids, particularly for PC, PE, and LPE. The traditional Folch method yielded a notably low content of these specific lipids, whereas the alternative approaches, even only with the use of ultrasound, resulted in a recovery almost three times higher than the conventional method. Although the impact on glycolipids was not as noticeable as for the phospholipids, as mentioned earlier, the eco-friendly alternatives still demonstrated a better recovery of DGDG and MGDG ($p < 0.05$ compared to the Folch method). Other interesting findings were in terms of ceramides and cholesterol esters. While an increasing trend was observed for the enzymatic mix in the recovery of ceramides, no significant differences were found between the other green methods tested ($p > 0.05$). Regarding cholesterol esters, Celluclast, Saczyme, and the enzymatic mix showed a better enrichment ($p > 0.05$ between the mentioned methods and $p < 0.05$ compared to the Folch method) of this specific compound class in microalgal extracts.

In general, we can conclude that the ultrasonic and enzymatic strategies are suitable and preferable alternatives to traditional chloroform-based methods (Folch method), as they offer not only higher extraction efficiency for all the targeted lipid classes of *M. gaditana* but also environmentally friendly and greener approaches for these valuable compounds.

4. Conclusion

In this work, we present an innovative strategy for (glyco-)lipidomics in *M. gaditana,* consisting of a green chemistry extraction approach, LC-HRMS(-MS) lipid profiling, and a comprehensive data evaluation workflow. We identified up to 520 lipid molecular species from 18 lipid classes, including polar membrane lipids and neutral storage lipids. Based on LC-MS, we could complement existing GC–MS profiles of *M. gaditana* by locating PUFA in the different microalgal lipid classes. Our results show that PUFA are mainly distributed in membrane lipids,

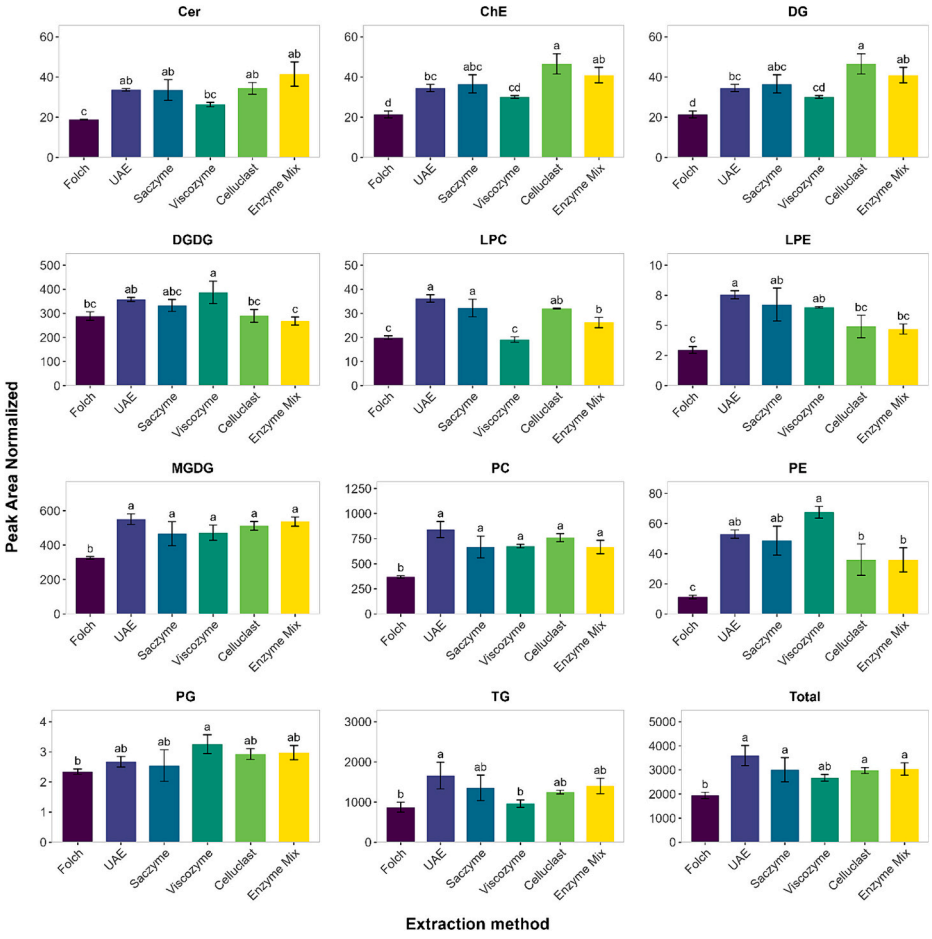

Fig. 5. Amount of lipids detected in each lipid class in the different *M. gaditana* extracts expressed as normalized peak areas. The peak area for each lipid species was normalized with the dry weight and class-specific standard. The bars represent the sum of normalized peak areas in each lipid class and sample. Treatments: Folch = Folch extraction; UAE = Ultrasound-assisted extraction (UAE) with ethanol; Saczyme = UAE with ethanol pre-treated with Saczyme; Viscozyme = UAE with ethanol pre-treated with Viscozyme; Celluclast = UAE with ethanol pre-treated with Celluclast; Enzyme Mix = UAE with ethanol pre-treated with an enzyme mix. The error bars present the standard deviation of three independent biological replicates ($n = 3$). Different letters specify statistically significant differences considering $p < 0.05$ (one-way ANOVA with Fisher LSD post-hoc test). Abbreviations: Cer = ceramides, ChE = cholesterol esters, DG = diglycerides, DGDG = digalactosyldiacylglycerols, LPC = lysophosphatidylcholines, LPE = lysophosphatidylethanolamines, MGDG = monogalactosyldiacylglycerols, PC = phosphatidylcholines, PE = phosphatidylethanolamines, PG = phosphatidylglycerols, PI = phosphatidylinositols, TG = triglycerides, Total = Total extracted lipidome.

in which cholesterol esters and polar lipids, namely PE, PG, and MGDG, have the highest PUFA content. These outcomes emphasize the importance of these structural and membrane lipid classes as a reservoir for PUFA in microalgae. Moreover, eco-friendly approaches allowed an improved extraction of lipids from microalgae while meeting health and environmental standards, such as the use of non-toxic solvents, which increases the value of the extracted compounds. In conclusion, this comprehensive analysis of the lipid composition of *Microchloropsis gaditana* significantly enhances our understanding of this microalgae species. By contributing valuable insights, it provides a crucial foundation for future research, unveiling pathways for the development of novel strategies to enrich or fractionate specific lipid classes. Simultaneously, this study introduces a potential tool for lipid profiling during biomass cultivation, highlighting its broader implications for the field.

CRediT authorship contribution statement

Marlene Pühringer: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Evelyn Rampler:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Natalia Castejón:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Summary data tables are found in the supplementary and raw data will be made available upon publication (ftp://massive.ucsd.edu/MSV000092532/).

Acknowledgments

This research was funded by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 847693. The authors thank the European Commission and the University of Vienna for the Marie-Curie REWIRE fellow granted to Natalia Castejón. The authors also thank Novozymes for

kindly providing the enzymatic solutions. The authors acknowledge the financial and scientific support of the (1) Department of Analytical Chemistry and (2) Department of Food Chemistry and Toxicology at the Faculty of Chemistry of the University of Vienna. We further thank all members of the Rampler, Koellensperger, and Marko labs for their great team spirit and scientific exchange. Open Access Funding is funded by the University of Vienna.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2024.103480.

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